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Parentage and relatedness determination in farmed Atlantic salmon (*Salmo salar*) using microsatellite markers

Ashie T. Norris *, Daniel G. Bradley, Edward P. Cunningham

Department of Genetics, Trinity College Dublin, Dublin 2, Ireland

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Abstract

This study demonstrates how both parentage and relatedness can be determined in a mixed family aquaculture (Atlantic salmon) stock in the absence of physical tags and/or pedigree information. Under a number of different scenarios, both real and simulated, we could use microsatellite markers to assign parentage to offspring with varying degrees of accuracy. The precision of assignment to one correct parental pair depended not only on the number and variability of the microsatellite markers, but also on the number of potential pairings from which to choose, i.e., the more families in the breeding program the more microsatellites required to discriminate between them. Using eight highly variable markers resulted in an assignment of an individual to the correct parental pair of greater than 95% even when the number of possible parent pairs is > 12,000. These same microsatellite loci were capable of discriminating between related and unrelated individuals in a situation where no pedigree information is known. Based on these results we conclude that the use of a number of microsatellite markers represents a realistic and cost-effective alternative to physical tagging in a family selection program.

Keywords: Parentage; Relatedness; Microsatellite markers; Atlantic salmon; *Salmo salar*

1. Introduction

One of the difficulties in implementing a selective breeding program in aquacultural stocks is maintaining pedigree information. Progeny from family groups must be

* Corresponding author. Tel.: +353-1-6083521; fax: +353-1-6798558; E-mail: anorris@tcd.ie

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isolated and reared together until they are large enough to be tagged. Not only is this approach space and labour intensive, but it also introduces environmental effects common to full-sib groups which are confounded with the genetic effects under study (Herbinger et al., 1999). Genetic profiles from microsatellite markers would allow retrospective assignment of individuals to family groups even after different progeny groups had been reared communally after hatching. Parentage assignment using genetic profiles has already been suggested for use in aquaculture situations in Atlantic salmon (Wright and Bentzen, 1994; Doyle and Herbinger, 1995). The feasibility of this approach has been assessed in communally reared rainbow trout, where 91% of fish were traced to one or two parental couples using four microsatellite loci (Herbinger et al., 1995) and in Atlantic salmon where between 80 and 99% of offspring were assigned to one set of parents in a number of different crosses using four microsatellite loci (O'Reilly et al., 1998).

Another difficulty in managing a selective breeding program is loss of genetic variability and increases in inbreeding as a result of the inadvertent mating of related individuals. The effects of inbreeding in salmonids include moderate to high inbreeding depression of survival and growth traits (Gjerde et al., 1983; Kincaid, 1983; Su et al., 1996). Inbreeding also will result in a decrease in genetic variability, which will limit the potential for genetic gain from artificial selection. Once reliable pedigree information is available, matings can be arranged that minimise inbreeding.

In the absence of pedigree information, as is likely in the first generation of a breeding program, the risk of mating related individuals may lead to inbreeding in families which form the basis of all selection in that population in the future. Estimates of relatedness based on DNA genotyping offer breeding managers of aquacultural stocks a method of avoiding inbreeding and maintaining genetic variation in the absence of pedigree information. Blouin et al. (1996) demonstrated the usefulness of microsatellite markers for discriminating unrelated from related individuals in a wild outbred population of mice using genotypes from 54 captured mice and randomly generated genotypes.

To address these issues, this study was initiated to investigate the feasibility of correctly assigning individuals to family groups using microsatellite data, and to assess the usefulness of these same microsatellites for estimating relatedness between pairs of individuals.

2. Materials and methods

2.1. Fish samples

The farmed populations used in this study were taken from an Atlantic salmon (*Salmo salar*) farm on the Northwest coast of Ireland. This Irish farmed population originates from a Norwegian strain, which was set up in the late 1960s from a number of wild Norwegian populations. Ova were imported into Ireland between 1982–1986 to create the Irish farmed population, and since then, the breeding stock has been closed.

A total random sample of over 200 individuals was taken from 5 year-classes of salmon (1993–1997). DNA from these individuals had already been genotyped at 15

microsatellite loci (Norris et al., 1999) (see Table 1). During the breeding season of 1997, tissue samples were taken from 12 broodstock (2 males and 10 females), which then were mated to create 10 full-sib and two half-sib families. Fertilised ova (~ 20) were taken from each pair mating before the ova were mixed so that the parentage of the offspring was known. These families were mixed, and once fry had emerged samples were taken from a further 25 offspring of unknown parentage.

2.2. Microsatellite analysis

Genetic profiles at all 15 loci were available for the Irish farmed salmon (Norris et al., 1999) (Table 1) and allele frequency distributions were obtained from these data. Family groups, which included parents and offspring, were assayed at eight loci (shown in Table 1). These loci were chosen for their ease of amplification and the ability to score them easily on autoradiographs of acrylamide gels. Due to difficulties in amplifying DNA from the fertilised eggs only these eight loci were used to genotype these family groups. Details of PCR conditions, variability in farmed salmon and references for all microsatellite loci are given in Table 1. PCR primers for loci *SSa141*, *SSa13*, *SSa132*, *SSa12*, *SSa129* and *SSa85* were designed from published sequences, and all other primers were synthesised according to referenced authors (see Table 1).

DNA was extracted from fin and muscle tissue samples by boiling samples in 500 μl solution A (200 mM NaOH) for 15 min and then adding 500 μl solution B (200 mM

Table 1
Details of the Atlantic salmon microsatellites used in this study

Microsatellite locus	$MgCl_2$ mM	Number of alleles[d]	Expected heterozygosity[d]	Annealing temperature (°C)	Reference/GenBank accession number
SSa141[a]	1.5	25	0.91	60	GenBank U58905
SSa13[a]	2.0	13	0.78	58	GenBank U58903
SSa132	1.5	5	0.42	55	GenBank U58901-
SSa12[c]	1.5	8	0.73	55	GenBank U58900
SSa129	1.5	3	0.15	54	GenBank U58899
SSa197[abc]	1.5	17	0.87	59	O'Reilly et al. (1996)
SSa171[abc]	2.0	13	0.84	58	O'Reilly et al. (1996)
SSa202[abc]	2.0	10	0.75	55	O'Reilly et al. (1996)
SSa85[abc]	2.0	14	0.88	57	O'Reilly et al. (1996)
SLOSL456	2.0	2	0.26	55	Slettan et al. (1997)
SLOSL444[a]	1.5	29	0.92	56	Slettan et al. (1995)
SLOSL439[ac]	1.5	22	0.89	58	Slettan et al. (1995)
SLOSL438	1.5	8	0.77	55	Slettan et al. (1995)
F43[c]	1.5	6	0.61	56	Sanchez et al. (1996)
20.19[c]	1.5	4	0.41	56	Sanchez et al. (1996)

[a] 8 Most variable microsatellite loci (used to generate progeny genotypes).
[b] 4 Microsatellite loci used to genotype parents and progeny in theoretical data set 1.
[c] 8 Microsatellite loci used to genotype parents and progeny in real data set and theoretical data set 1.
[d] Figures come from genotypes of over 200 farmed salmon previously profiled at all loci.

HCl, 100 mM Tris–HCl, pH 8.5). Approximately 20 fertilised ova were taken from each family, and DNA was extracted as described in Slettan et al. (1997). Polymerase chain reactions were performed in 5 μl reaction volume containing 0.55 μl Taq polymerase buffer (100 mM Tris–HCl [pH 9.0], $MgCl_2$ concentrations as given in Table 1, 1% Triton X-100, 500 mM KCl), 0.3 mM primer A, 0.3 mM primer B, 200 mμ of each dGTP, dTTP, dATP, 20 μm dCTP, 0.1 mμ α-^{32}P dCTP (3000 Ci/mmol), 0.5 units Taq polymerase and H_2O to 5 μl. Temperature cycling conditions were as follows: one denaturation at 94°C for 3 min; 38 cycles of 45 s at 94°C; 45 s at annealing temperatures given in Table 1; 45 s at 72°C and a final extension at 72°C for 4 min. PCR products were electrophoresed in 6% acrylamide and autoradiographed.

2.3. Statistical analysis

Expected heterozygosities for all loci and populations were estimated according to (Nei, 1978). Likelihood of pedigree relationships was assessed using a program, Kinship version 1.2, developed by Queller and Goodnight (1989). This program tests the hypothesis of a specified pedigree relationship given the population allele frequencies. After generating a likelihood that the specified relationship is true, Kinship estimates a significance level for this likelihood value by generating random pairs of individuals and determining the likelihood value needed to reject the null hypothesis at different significance levels. Parent-offspring pairs showing significant likelihood values ($P < 0.05$) are then used in a further analysis where all possible maternal and paternal identifications are included in every combination. Kinship will treat that identification as part of the hypotheses being tested, rejecting the hypotheses for any parental pair that cannot actually be the parents of the individual being tested (even though individually they could have been). Individuals were thus assigned to parent pairs.

The same program was used to generate the relationship statistic r_{xy} of Queller and Goodnight (1989), which is an unbiased estimate of the true genetic relationship between individuals x and y. The r_{xy} statistic is considered, by the authors, to be preferable to an allele sharing statistic as it is a continuous variable and utilises the population allele frequencies (already estimated) to eliminate bias due to small sample sizes.

Ten full-sib and two half sib families were available for parentage analysis. However, in a real breeding program parentage for offspring will have to be assigned from a pool of many more parents. In order to simulate this situation, genotypes of over a further 200 salmon from the same population were included in the analysis. These individuals represented virtual parents as a background to the 12 actual parents. Three data sets were therefore available for analysis.

Real data set: Genotypes from approximately 16 eggs from the 10 family groups and from 25 mixed fry were assayed at eight loci. These offspring genotypes first were compared with genotypes from their real parents in order to confirm known pedigree information on all families.

Theoretical data set 1: Next, the microsatellite genotypes of these offspring were compared with their real parents and with 200 other farmed salmon from the same population (previously genotyped at 15 microsatellite loci) (Norris et al., 1999) in order to estimate the accuracy with which individuals now can be assigned exclusively to their

real parents. In this scenario we assumed that as well as the 12 real parents (10 pairs of parents), there were 200 other potential parents from the same population which were grouped into 100 hypothetical pairs (100 assigned as females and 100 assigned as males) giving 110 pairs of parents. Genotypes from both eight and four microsatellite loci were used for this analysis (see Table 1).

Theoretical data set 2 with factorial mating: Further discrimination may be required where there are more than 110 pairs in the family population. In order to demonstrate this, family groups (10 pairs of parents with 20 offspring each) were generated from simulated pairings of salmon previously genotyped at all loci. Two sets of simulated pairings were done, one using all 15 loci and the other using the eight most variable loci (see Table 1). Offspring from these simulated pairings were not only compared to the 110 pairs of parents as before, but were now compared with all possible combinations of these parents giving over 12,000 potential parent pairs.

For both relatedness and parentage estimations, genetic profiles from all parents and offspring, from 10 family groups, were available for eight microsatellite loci (see Table 1). Computer simulated genotypes were generated for a further generation of individuals. The mating structure assumed that individuals of the actual full-sib progeny were mated to unrelated partners. Thus, their offspring are first cousins. Genotypes of simulated individuals were generated using the module PROBMAXG of the program PROBMAX (Danzmann, 1997). In addition, genetic profiles from randomly chosen, unrelated salmon at 15 microsatellite loci, including the eight most variable loci, were used to generate genotypes of full-sib, half-sib and cousin groups using the same program.

Relatedness values between individuals based on actual genotypes at all loci were available for individuals across and between year-classes. For the purposes of this study, this group will be termed 'unknown relatedness' as it may contain related individuals. To estimate the probability that an individual might be placed in the wrong group, a criterion was chosen for each group below or above which any individuals would not be classified as belonging to that group.

3. Results

3.1. Matching offspring to parents

Based on the output from the Kinship program there are a number of different outcomes for each individual: an individual may be assigned to no parent pair, an incorrect parent pair, the correct parent pair and no others, or more than one pair. Using the real data set, all but 5, individuals, out of 160, were exclusively assigned to their correct parent pair. One individual was assigned to two parental pairs both of which could have been the parents. A further four individuals had genotypes inconsistent with any parental cross and having confirmed that these were not scoring errors, were subsequently genotyped a second time. Inconsistent genotypes such as these may occur due to a number of reasons: eggs may be mixed between families in the hatchery or in the laboratory, samples may be inadvertently mixed between wells during DNA

extraction and/or PCR, or a mutation may occur in the germ line of the parents or in the developing embryos themselves. Having re-genotyped all samples with inconsistencies, two were confirmed as being mutations (in microsatellite loci *SSa202* and *SSa171*) and the other two were a result of contamination during PCR and were later matched to their correct parents. The two mutations were detected over 320 meioses (160 offspring) and eight loci giving a mutation rate of 7.8×10^{-4} or 1 in 1280. This is twice as high as the mutation rate observed in Atlantic salmon by O'Reilly et al. (1998). However, this difference does not seem significant given the small sample in this study. In the case of the 25 fry, all individuals were unambiguously matched to one set of parents.

Scoring errors were not a major source of error in this analysis as, with the exception of the 25 mixed fry, all individuals were genotyped alongside their real parents, thereby ensuring ease of allele scoring. In a study where parentage was determined for communally reared offspring, O'Reilly et al. (1998) found a scoring error rate of 2–3% per allele scored. However, this group found that 90% of these errors were later detected and only 0.5% would lead to an incorrect assignment of offspring to parent.

Using the theoretical data set, all offspring, except the two individuals mentioned above, were assigned to either one or two parental pairs. Table 2 shows the breakdown of allocation of all individuals to parental pairs in this scenario. In total, 95.6% of individuals could be traced uniquely to their real parents out of a possible 110 pairs of parents. A further 3.3% could be narrowed down to two sets of parents. Overall, 98.9% of individuals could be allocated to, at most, two parental pairs and the remainder, as a result of mutations, could not, unless the mutated locus was eliminated from genotype, be matched to any parental pair. The use of four microsatellite loci to genotype parents and offspring resulted in the percent of offspring correctly assigned to one parent pair reduced to 94.3% (Table 2).

Allocation to a single pair could be improved further with the replacement of one or two of the less variable markers used in the study by more variable ones. This further discrimination would be required where there are more than 110 sets of potential parents

Table 2
Assignment of offspring in parentage study under three different scenarios

Result	Genotypes from:			
	8 Microsatellite loci[a]	4 microsatellite loci[a]	15 microsatellite loci[b]	8 most variable microsatellite loci[b]
% of offspring assigned to true parents and no others	95.6%	94.3%	100%	98%
% of offspring assigned to 2 sets of parents (including true parents)	3.3%	4.6%	0%	2%
% of offspring wrongly assigned or assigned to no parental pair	1.1%	1.1%	0%	0%

[a] Theoretical data set 1. Percentages are given as number of offspring out of 185.
[b] Theoretical data set 2. Percentages are given as number of offspring out of 200.

in the family population. Theoretical data set 2 demonstrates the discrimination required when a larger number of parent pairs are involved in the analysis. The results from these generated progeny sets can be seen in Table 2. Using 15 loci ensured all 200 offspring were traced to their correct parental pair and to no other possible pair. When genotypes were generated using the eight most variable loci this figure was reduced to 196 with four individuals having two possible sets of parents (one correct set and one other).

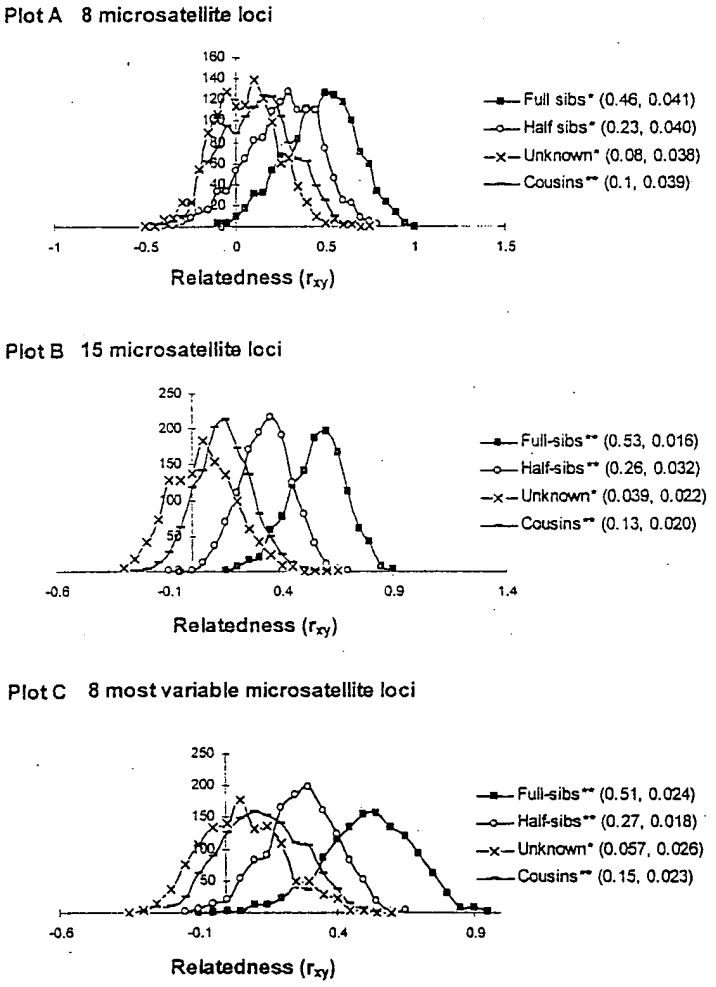

Fig. 1. Relatedness values for full-sibs, half-sibs, cousins and individuals of unknown relatedness under three different screening strategies. Mean and standard deviation of distributions are shown in parentheses. The 'unknown' group refers to individuals sampled both within and between year classes and, therefore, could contain full- and half-sibs.

* Actual genotypes. ** Randomly generated genotypes.

3.2. Relatedness

A relatedness statistic, r_{xy}, (Queller and Goodnight, 1989) was calculated for all parents and offspring which had been genotyped at the eight loci shown in Table 1 and for all other farmed salmon of unknown relatedness which had been genotyped at all 15 loci. The same calculations were performed for individuals whose genotypes had been generated by random simulation. Distributions of r_{xy} for the three scenarios mentioned above are shown in Fig. 1. Using individuals which had been actually genotyped at eight loci (plot A) results in distributions with higher standard deviations for each group than for the other two scenarios. The means and standard deviations for r_{xy} for all related groups in plot A are all somewhat lower than would be expected for full-sibs, half-sibs and cousins. This may be a sampling effect, as the 95% confidence levels for these means are all just above 0.01, or it may be due to the low power of these loci for detecting relatedness. The difference in the standard deviation of the distributions between plot A and plot B is not observed between plot B and plot C, indicating that the same or similar resolution is being obtained with the eight most variable microsatellite markers as with 15. The 'unknown' groups here consist of individuals sampled both between and within year classes of farmed salmon and, therefore, could contain full and half-sibs. This explains the right skew of relatedness values for this group in all plots. Where genotypes of offspring were generated by simulation from randomly chosen salmon previously genotyped, the relatedness values were all significantly higher ($P < 0.01$) than would be expected for full-sibs, half-sibs and cousins (plots B and C). This may be a result of generating offspring from parents which may already have been distantly related.

In an aquaculture situation, the purposes of estimating relatedness among potential mating pairs is to avoid mating related individuals in the absence of pedigree information. Since salmon broodstock are slaughtered after spawning, no potential parent–offspring pairs will exist, and the related pairs most likely to be found are full-sibs, half-sibs and cousins. The objective of using microsatellite markers is to allow us to classify all potential pairs as either related or unrelated. There are two types of errors in misclassification. A pair may be misclassified as related when they are in fact unrelated

Table 3
Misclassification rates under the three different scenarios

Misclassification type	Genotypes from:		
	8 Microsatellite loci	15 Microsatellite loci	8 Most variable microsatellite loci
Proportion misclassified using mean of distribution of unknown relatedness as cut-off point			
Full-sibs classified as unrelated	0.05	0.00	0.01
Half-sibs classified as unrelated	0.22	0.01	0.08
Cousins classified as unrelated	0.46	0.26	0.28
Proportion misclassified using 0 relatedness as cut-off point			
Full-sibs classified as unrelated	0.01	0.00	0.00
Half-sibs classified as unrelated	0.13	0.00	0.01
Cousins classified as unrelated	0.33	0.07	0.08

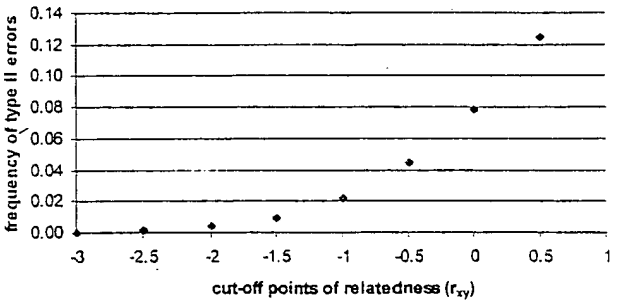

Fig. 2. Frequency of type II errors (related individuals being classified as unrelated) under relatedness criteria between 0.5 and −3.0. Lowering the criterion will result in fewer related individuals being classified as unrelated but will also result in fewer pairs being available for selection on the basis of relatedness.

(type I error) or a pair may be misclassified as unrelated when they are in fact related (type II error). Type II error is the one we are most concerned with as this error could result in related pairs being mated.

The frequency of both types of errors will depend not only on the number and variability of microsatellite loci used but also on the criteria of relatedness chosen below which all pairs will be classified as unrelated. Table 3 shows the proportion misclassified as type II errors given each scenario using two different values of relatedness as criteria. Using 15 microsatellites greatly improves the misclassification rates. However, using the eight most variable loci and the stricter decision criterion discriminates between related and unrelated almost to the same extent. Lowering the criterion improves the classification rates by allowing fewer related pairs to be classified as unrelated. By using 0 instead of the mean of pairs of unknown relatedness results in just over 1 in 10 cousins and 1 in 100 half-sibs being classified as unrelated (Table 3).

The effect of lowering the criterion even further can be seen in Fig. 2 where the frequency of type II errors (for all related individuals) was estimated using criteria ranging from 0.05 to −0.3 and using the eight most variable microsatellite loci. However, using a lower criterion will result in fewer pairs being classified as unrelated and will, therefore, limit the number of pairs available for selection. For example, using 0 relatedness as the criterion would result in just over 40% of all potential mating pairs being categorised as unrelated, and lowering it further to −0.15 reduces this percentage to 13%. Where relatedness, based on microsatellite markers, is being used to avoid mating related individuals, a balance needs to be found between lowering the criterion of selection and ensuring that sufficient pairs are available for selection.

4. Discussion

This study demonstrates that both parentage and relatedness can be determined in the absence of physical tags and pedigree information in a mixed farmed Atlantic salmon population. Using a small family group of 10 full-sib families, 98% of offspring were unambiguously assigned to one correct set of parents. In a breeding program, however,

the number of family groups will likely be between 100–200 and a greater resolution would be required to discriminate between groups. Using the real parent group as well as another 100 possible parents resulted in a reduction in the discrimination to 95.6% using eight microsatellite loci and 94.3% using four microsatellite loci. This figure is intermediate between the figures presented by O'Reilly et al. (1998) for the same microsatellites for 36 full-sib families (99.6%) and 144 full-sib families were included (81.6%). It is interesting to note that increasing the number of microsatellites from four to eight results in very little extra discrimination, this is likely due to the fact that the four extra markers have low variability in our population.

Ideally, it would be preferable to attain a higher correct assignment rate as any error will result in performance information being credited to the wrong families. Adding an extra seven markers to the simulated data set results in 100% correct assignment, even when the number of possible parent pairs is $> 12{,}000$. However, we would like to achieve the same or similar assignment from a smaller number of microsatellite markers. This is important in order to make genetic profiling a realistic alternative to physical tagging from both a practical and economic point of view. When the eight most variable loci (average expected heterozygosity = 0.85) were used to generate genotypes of family groups, the correct allocation was reduced by only 2%. This discrimination is sufficient for parentage purposes given that in a real breeding program the number of parental combinations is unlikely to be higher than 300. From both theoretical data sets we can see that it is the variability of the microsatellite loci in the particular population that is of most important for discrimination. For practical purposes therefore, a trade-off will be required between the acceptable number of genotypes unresolved (or assigned to only one parent) and the cost and time of genotyping.

The same microsatellite markers also were shown to be capable of discriminating between related and unrelated salmon in a situation where no pedigree information is available. As in the parentage study, using 15 loci gives the maximum accuracy, but the use of eight most variable loci results in a decrease in accuracy small enough to be tolerated for the sake of time and money saved in genotyping.

Using genetic profiling for either parentage and/or relatedness purposes also will allow a breeding manager to monitor changes in genetic variation each generation that may result from a family-based selection program. Norris et al. (1999) showed significant differences in genetic variation between major farmed salmon stocks, which could be a result of differences in selection programs between these farms. Microsatellite-based genetic profiling has been shown here as having the potential to replace traditional procedures for the identification of families in a salmon aquaculture breeding program. This would eliminate the need to rear families separately until they can be physical tagged and at the same time provide a method to minimise increases in inbreeding that may result from a genetic improvement program.

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